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19 ABSTRACT (Continue on reverse if necessary and identify by block number) Endotoxin associated proteins (EP) from <u>Bordetella pertussis</u> and <u>Salmonella typhi</u> have been found to be potent immunomodulators. EP is an adjuvant for T-dependent and T-independent antigens. Both the primary and secondary antibody responses to T-dependent antigens are enhanced. T-cells and macrophages act as accessory cells in this adjuvant effect; however, a significant portion of the enhanced antibody response to any one antigen is due to polyclonal activation by EP. In addition EP activates macrophages to produce the lymphokine Interleukin-1 which may play a role in the adjuvant effect as well. Finally, lipopolysaccharide endotoxin derived from <u>Bordetella pertussis</u> has been found to activate the B-cells and macrophages of C3H/HeJ mice which are hyporesponsive to LPS endotoxin from enteric Gram-negative bacteria.			
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Certain outer membrane proteins, which can be extracted in complex form associated with the lipopolysaccharide (LPS) endotoxin of Gram-negative bacteria, have been found to be potent activators of lymphocytes and macrophages (Sultz, 1983). These proteins, which we have referred to as endotoxin protein (EP), are separable from the LPS by hot phenol-water extraction and have the property of collectively and individually activating the proliferation of B-cells from LPS hyporesponsive C3H/HeJ mice (Sultz and Goodman, 1976). In addition, EP stimulates polyclonal antibody synthesis, activates macrophages to be cytotoxic for tumor cells, and induces interferon activity in mice (Sultz, 1983). More recently, we have also found that EP from Salmonella typhi (TEP) and Bordetella pertussis (PEP) can act as adjuvants (Sultz et al., 1985). This report will amplify the evidence of the immunoregulatory activities of EP and, furthermore, describes an unanticipated finding that pertussis LPS endotoxin can activate C3H/HeJ lymphocytes and macrophages.

A. Adjuvant activity of EP in vivo.

1. Enhancement of antibody to sheep red blood cells (SRBC). Using SRBC as a classic example of a T-dependent antigen, mice were immunized intravenously with this antigen at a concentration of 0.5% SRBC. To this suspension was added 10 µg of TEP or PEP and the IgG response was then measured by the plaque forming cell assay (PFC) 7 and 14 days after the initial injections. The results shown below (Table 1) demonstrate a significant enhancement of the normal response to SRBC at both time points by the inclusion of either adjuvant. The efficacy of these materials is emphasized by the fact that all immunizations were accomplished by a single injection with solubilized adjuvant. No oils or any other vehicles were added to produce depot responses.

2. Enhancement of antibody to polysaccharide antigens. To determine whether EP could adjuvant a so-called thymus-independent antigen, pneumococcal polysaccharide S3 was given simultaneously with TEP or PEP by the intraperitoneal route. PFC assays for IgM antibody were made from the splenic lymphocytes of CF-1 mice at an optimal time for the IgM response to pneumococcal polysaccharides in mice. As shown in Table 2, the enhancement of IgM - PFCs by both TEP and PEP was detected at high and low doses of antigen. Of additional interest is the fact that when Salmonella typhi LPS was given under the same regimen, a significant depression from 70-88% in IgM PFCs was obtained. Thus under these experimental conditions, EP does not serve to suppress antibody production to polysaccharides as is the case with LPS.

3. The kinetics of the adjuvanticity of EP with cholera toxoid. Previous experiments have clearly shown that TEP and PEP adjuvant the immune response to cholera enterotoxin in mice after immunization with cholera toxoid (Sultz et al., 1985). In these experiments, increased protection in mice challenged with lethal toxin correlated with higher antitoxin levels at the time of challenge some 6 weeks after the mice were immunized. An extension of this experiment was undertaken to investigate the levels of neutralizing antitoxin levels in the serum of mice 2 to 10 weeks after the animals were immunized with cholera toxoid and either whole cell pertussis vaccine or PEP as adjuvants. Antitoxin levels were measured using the Y-1 adrenal cell



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toxin assay. Representative results of this experiment are shown in Table 3. Of particular interest is the fact that the adjuvant effect of PEP is highly significant as early as 2 weeks after a single dose in combination with cholera toxoid. At 10 weeks post immunization the enhancement is even greater so that the cholera toxoid dose needed to elicit 1.0 antitoxin unit per ml of serum was reduced some 140 fold. Although the experiment was terminated at 10 weeks, the data suggest that no endpoint in the adjuvant effect was reached. In addition, it should be noted that PEP compares favorably with the whole cell vaccine which contains other adjuvant materials aside from EP. Finally, from other data (not shown), PEP stimulates antibody production at doses of cholera toxoid which are ineffective alone.

#### B. Immunoregulatory Activities of EP at the Cellular Level.

1. Enhancement of secondary responses to antigen. In previous studies, the stimulation of antibody synthesis to cholera toxoid and SRBC in primary lymphocyte cultures by EP has been demonstrated to equal or surpass the potent adjuvant effect of LPS (Sultz et al., 1985). When splenic lymphocytes derived from mice primed with SRBC are used in culture, the secondary PFC response can be enhanced as well (Table 4). The optimal IgM response is obtained when both the antigen and adjuvant are cultured with cells primed with both SRBC and the TEP adjuvant. The enhancement of IgG PFCs to SRBC using the same protocol as shown in Table 4 also has been obtained.

2. The role of accessory cells in the adjuvant effect. The requirement for B-cell interaction with T-cells and/or macrophages in the EP adjuvant effect was examined using lymphocyte cultures depleted of each cell type. The removal of T-cells by monoclonal anti-thy 1.2 antibody plus complement altered the response to cholera toxoid as measured by the direct PFC response to cholera toxin coated SRBC. As shown in Table 5, 50 to 60% of the PFC response was lost in both CBA/J and C3H/HeJ lymphocyte cultures when the T-cells were depleted; however, the significant polyclonal stimulation by TEP alone to cholera toxoid was unaffected. In a similar manner, depletion of macrophages by repeated carbonyl iron treatment of spleen cells (approximately 1% residual macrophages) reduced the adjuvant response for TEP and PEP by 85% and 71% respectively (Table 6). Nevertheless, the direct polyclonal stimulation by TEP and PEP to cholera toxoid remained essentially intact. It is our conclusion, therefore, that T-cells and macrophages play a significant accessory role in the adjuvanticity generated by EP as measured in vitro. Furthermore, a substantial portion of the overall enhancement of the response, at least to the T-dependent antigen cholera toxoid, is due to polyclonal activation which is not affected by the removal of macrophages or T-cells.

3. Interleukin-1 activity stimulated by EP. In view of the role of macrophages as accessory cells for the adjuvant effect of EP and since EP has been shown to activate mouse macrophages to be cytotoxic for tumor cells, we investigated the possibility that macrophage Interleukin-1 (IL-1) activity could be stimulated by EP. For this purpose, resident peritoneal macrophages from normal mice were collected, plated and cultured in the presence of each stimulant at 100 µg per culture. After twenty-four hours, the culture supernatants were harvested and assayed for IL-1 activity by the C3H/HeJ

thymocyte assay. The results (Fig. 1) clearly show that supernatants synergize with Concanavalin A to stimulate thymocyte proliferation. We conclude, therefore, that EP like LPS can stimulate IL-1 activity from mouse macrophages which may very well contribute to the adjuvanticity of EP.

#### C. Immunogenicity of PEP.

Since the polypeptides of EP are found in the outer membrane of Gram-negative bacteria, we reasoned they may act as protective immunogens. Our previous studies demonstrated that EP from Salmonella typhimurium protected CD-1 mice against a challenge of 500 LD50's of S. typhimurium, was more effective than LPS and at the same time relatively non-toxic. Our experience with B. pertussis EP, using the standard protection assay prescribed for testing the efficacy of the whole cell pertussis vaccine currently used in the United States, indicated that the trichloroacetic acid extract (Boivin antigen) containing both LPS and outer membrane proteins (EP) was highly protective. In addition, live or dead whole cells of B. pertussis injected into mice produced high titers of antibody to PEP as measured by the ELISA assay. This indicated that the antigenic determinants of PEP are revealed to the host and could possibly serve as protective immunogens (see Annual Report 1984-85).

In more recent studies, PEP and pertussis LPS were dissociated from the Boivin antigen by hot phenol-water extraction and purified by alcohol precipitation and ultracentrifugation. Both major components were tested by the protection assay either individually and/or together. However, neither the EP or the LPS at a wide range of doses protected the mice against the challenge infection with B. pertussis. This result was not unexpected with LPS since the LPS is known not to be a protective antigen; however, the possibility of a synergy between LPS and EP to explain the high degree of resistance provided by the pertussis Boivin antigen was also ruled out by the fact that various dosage combinations of LPS and EP did not increase the resistance of the mice to the standard challenge. Consequently, we have concluded that in the process of purifying the PEP, we have lost or denatured the protective antigen(s) which remains undefined as yet.

#### D. Activation of LPS hyporesponsive cells by pertussis LPS.

As a by-product of the extraction of PEP, we recovered LPS using the phenol-water method (PW) for purifying LPS from the Boivin endotoxin. The LPS recovered contained less than 1% protein and was mitogenically inactive on C3H/HeJ splenic lymphocytes (Sultz et al., 1985). However the yields were relatively small and since pertussis LPS is similar to rough (R) enteric LPS, we adopted the phenol-chloroform-petroleum ether (PCE) method (Galanos et al., 1969) which efficiently extracts R-LPS. By this procedure, we obtained essentially protein-free pertussis LPS which, surprisingly, was active as a mitogen for C3H/HeJ spleen cells (Table 7). In fact the activity of PLPS is amplified when C3H/HeJ B-cells are used (Table 8), whereas the typical enteric LPS or the PW-LPS is essentially inactive. As expected, all of the LPS preparations are active on the LPS responsive CBA/J and C3H/OuJ strains, regardless of the source or extraction method used. Furthermore, the PCE-LPS activated polyclonal antibody synthesis in C3H/HeJ B-cells (Table 9) and stimulated deficient C3H/HeJ macrophages to secrete Interleukin-1 (Fig. 2).

When these pertussis LPS preparations were analyzed by SDS-PAGE, a clear difference was obtained (Fig. 3). The PW-LPS demonstrated one major band, whereas the PCE-LPS produced 2 major bands (1,2) a diffuse area of staining between the major bands (3), and the suggestion of a faster-migrating minor band (4). From these results, it appears that the major species (2) is inactive on C3H/HeJ B-cells and the slowest migrating species (1) is most likely responsible for the mitogen effect observed with C3H/HeJ cells. Indeed, in a preliminary experiment wherein we have separated the two major molecular species of the PCE-PLPS by hydroxylapatite chromatography, (1) is mitogenic for C3H/HeJ spleen cells whereas (2) is not, although both components activate C3H/OuJ cells.

Polymyxin B inhibition experiments using lipid-enriched fractions of LPS produced by acetic acid hydrolysis revealed that the mitogenic activity of the pertussis glycolipid fraction could be significantly reduced. As shown in Table 10, a comparable reduction in activity is evident in the cultures from both C3H/OuJ and C3H/HeJ mice in the presence of a PB suggesting that the lipid of pertussis LPS, at least in part, is responsible for the mitogenic activity on C3H/HeJ cells.

Taken together, these data provide evidence to suggest that a specific molecular species of pertussis LPS has the property of activating C3H/HeJ B-cells, which are ordinarily unresponsive to the lipid-A from enteric LPS. Further studies on the chemistry of PLPS may provide an insight into the nature of the deficiency of C3H/HeJ B-cells in terms of their response to various lipids derived from lipopolysaccharide endotoxins.

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**Table 1.** Adjuvant Effect of Endotoxin Protein on IgG Response to Sheep Erythrocytes in CBA/J Mice.

Mean No. Indirect PFC/10 <sup>6</sup> Spleen Cells <sup>1</sup> .				
Antigen/Adjuvant <sup>2</sup> .	Exp. 1		Exp. 2	
	7 Days	14 Days	7 Days	14 Days
SRBC	89	22	56	35
SRBC + TEP	439 (4.9) <sup>3</sup> .	157 (7.1)	315 (5.6)	109 (3.1)
SRBC + PEP	590 (6.6)	163 (7.4)	297 (5.3)	140 (4.0)

1. 4-5 Mice per group.

2. SRBC (0.5%) given i.v. with adjuvant (10 µg).

3. ( ) Stimulation Index.

**Table 2.** Enhancement of IgM Antibody to S3 Pneumococcal Polysaccharide in Mice by Endotoxin Protein.

Total Mean PFC/Spleen <sup>2</sup> .		
Antigen/Adjuvant (µg) <sup>1</sup> .	at 5 Days	% Increase
S3 (0.1)	3,178	-
+ TEP (10)	5,106	60.7
+ PEP (10)	5,459	71.8
S3 (1.0)	5,898	-
+ TEP (10)	10,903	84.9
+ PEP (10)	8,761	48.5

1. Antigen and Adjuvant given simultaneously by i.p. route.

2. Direct plaques, mean of 4 mice per group. (CF-1).

Table 3. Enhancement by PEP Adjuvant of the Cholera Antitoxin Response in Mice.

Antigen/Adjuvant	Effective Toxoid Dose <sup>1</sup> . (μg) at	
	2 weeks	10 weeks
CTd <sup>2</sup> .	0.50	1.69
CTd/WCV <sup>3</sup> . (0.05 ml)	0.24 (2.1) <sup>4</sup> .	0.006 (282)
CTd/WCV (0.25 ml)	0.006 (83.3)	0.059 (28.6)
CTd/PEP (2 μg)	0.018 (27.8)	0.030 (56.3)
CTd/PEP (10 μg)	0.013 (38.5)	0.012 (141)

1. Estimated effective cholera toxoid dose required to elicit 1.0 Antitoxin Unit per ml. All injections given intraperitoneally.
2. CTd = cholera toxoid
3. WCV = whole cell B. pertussis vaccine
4. ( ) = Fold-increase over CTd given alone.

Table 4. Adjuvant Effect of Endotoxin Protein on Cultures of Antigen Primed Lymphocytes<sup>1</sup>.

Cultured With <sup>1</sup> .	<u>Primed With</u>			
	<u>SRBC</u>		<u>SRBC + TEP</u>	
	CBA/J	CF-1	CBA/J	CF-1
0	7 <sup>2</sup> .	246	5	228
SRBC	22	1367	49	440
SRBC + TEP	963 (43.8) <sup>3</sup> .	3261 (2.4)	2708 (55.3)	2626 (5.9)

1. Splenic lymphocytes cultured 5 days after priming injection.
2. Mean no. of direct PFC/10<sup>6</sup> spleen cells after 5 days of culture.
3. ( ) Fold-increase over mice primed with SRBC or SRBC + TEP.

Table 5. Effect of T-cells on the Adjuvanticity of EP in Murine Lymphocyte Cultures<sup>1</sup>.

Antigen/Adjuvant	Mean No. PFC/10 <sup>6</sup> Spleen Cells					
	CBA/J			C3H/OuJ		
	s.c.	s.c.-t <sup>4</sup> .	(change)	s.c.	s.c.-t <sup>4</sup> .	(change)
None	15	2		0	9	
PCG <sup>2</sup> .	6	0		60	9	(- 85)
TEP <sup>3</sup> .	415	615	(+ 48)	380	395	(+ 4)
PCG + TEP	1590	620	(- 61)	520	250	(- 52)

1. 6 days of culture, direct PFCs measured against cholera toxin coated SRBC.
2. Procholeragenoid antigen at 1 µg/ml of culture.
3. TEP at 10 µg/ml of culture.
4. Depletion by monoclonal anti-Thy 1.2 plus c' varied from 86% to 100% as measured by Con A response assay.

Table 6. Effect of Macrophages on the Adjuvanticity of EP in Murine Lymphocytes Cultures<sup>1</sup>.

Antigen/Adjuvant	Mean No. PFC/10 <sup>6</sup> Spleen Cells		
	Undepleted	Depleted <sup>2</sup> .	(% change)
None	0	0	
PCG <sup>3</sup> .	0	0	
TEP <sup>4</sup> .	177	292	(+ 65)
PCG + TEP	569	85	(- 85)
PEP <sup>4</sup> .	272	238	(- 13)
PCG + PEP	1450	417	(- 71)

1. 6 days of culture, direct PFCs measured against cholera toxin coated SRBC.
2. Macrophage depletion by carbonyl iron method.
3. Procholeragenoid antigen at 1 µg/ml of culture.
4. TEP and PEP at 10 µg/ml.



Table 7. Stimulation of DNA Synthesis of C3H/HeJ Splenic Lymphocytes by Pertussis Lipopolysaccharide<sup>1</sup>.

LPS, $\mu$ g/culture		Mean Net cpm of <sup>3</sup> H-Tdr Incorporation	
<u>Exp. 1</u>		<u>C3H/HeJ</u>	<u>CEA/J</u>
PLPS (PW) <sup>1</sup> .	1	98	42,433
	10	767	43,350
	100	1,086	9,927
<u>Exp. 2</u>		<u>C3H/HeJ</u>	<u>C3H/OuJ</u>
PLPS (PCE) <sup>2</sup> .	0.1	3,683	36,920
	1.0	10,966	23,294
	10.0	16,199	10,622

1. Pertussis LPS (PW) extracted by phenol-water method.

2. Pertussis LPS (PCE) extracted by phenol-chloroform-petroleum ether method.

Table 8. Stimulation of DNA Synthesis of C3H/HeJ B-Enriched Splenic Lymphocytes by Pertussis Lipopolysaccharide Endotoxin.

LPS, $\mu$ g/culture		Mean Net cpm of $^3\text{H}$ -Tdr Incorporation			
Spleen Cells		C3H/HeJ	(SI)	C3H/OuJ	(SI)
PLPS <sup>1</sup> .	0.1	11,666	(7.0)	49,688	(5.3)
"	1.0	8,636	(5.5)	41,583	(4.6)
"	10.0	5,847	(4.0)	37,781	(4.2)
TLPS <sup>2</sup> .	0.1	439	(1.2)	35,581	(4.1)
"	1.0	541	(1.3)	35,736	(3.9)
"	10.0	607	(1.3)	23,990	(3.1)
<u>B-cells<sup>3</sup>.</u>					
PLPS	0.1	6,900	(3.9)	50,798	(9.5)
"	1.0	13,680	(6.8)	58,155	(10.9)
"	10.0	30,230	(13.8)	53,729	(10.0)
TLPS	0.1	459	(1.2)	42,716	(8.2)
"	1.0	830	(1.4)	49,549	(9.3)
"	10.0	650	(1.3)	22,860	(4.8)

1. Pertussis LPS extracted by phenol-chloroform-petroleum ether. No protein detected (Lowry).
2. LPS from *S. typhi* 0-0901 by phenol water method. Less than 1.0% protein (Lowry).
3. Spleen cells depleted of T-cells by monoclonal anti-Thy 1.2 and c' (96% depletion by Con A assay).
4. (SI) = Stimulation index, Experimental cpm  $\div$  Control cpm.

Table 9. Polyclonal Antibody Synthesis Stimulated in C3H/HeJ Lymphocytes by Pertussis Lipopolysaccharide.

Stimulant ( $\mu$ g)	Mean No. PFC <sup>1</sup> /10 <sup>6</sup> Spleen Cells	
	C3H/OuJ	C3H/HeJ
None	25	10
PEP <sup>2</sup> . (10)	550	215
" (50)	1085	380
" (100)	650	610
PLPS <sup>3</sup> . (10)	625	150
" (50)	685	175
" (100)	595	190

1. Plaque-forming cells against TNP-SRBC after 72 hrs of culture.
2. Pertussis endotoxin protein.
3. Pertussis lipopolysaccharide extracted by the phenol-chloroform-petroleum ether method.

**Table 10.** Effect of Polymyxin B on the Stimulation of DNA Synthesis in Splenic Lymphocytes by Pertussis LPS.

Mitogen ( $\mu$ g)	Mean Net cpm of $^3\text{H}$ -Tdr-Incorporation	
	C3H/OuJ	C3H/HeJ
PL <sup>1</sup> , 0.1	36,429	8,661
" , 1.0	57,016	14,542
PL, 0.1 + PB <sup>2</sup> .	10,828 (70%) <sup>3</sup> .	1,611 (78%)
" 1.0 + PB <sup>2</sup> .	37,607 (34%)	9,018 (38%)

1. PL = Pertussis glycolipid from acetic acid hydrolyzed pertussis LPS according to the method of Le Duc, et al. 1980.
2. PB = Polymyxin B at 10  $\mu$ g per culture.
3. (%) percent inhibition.

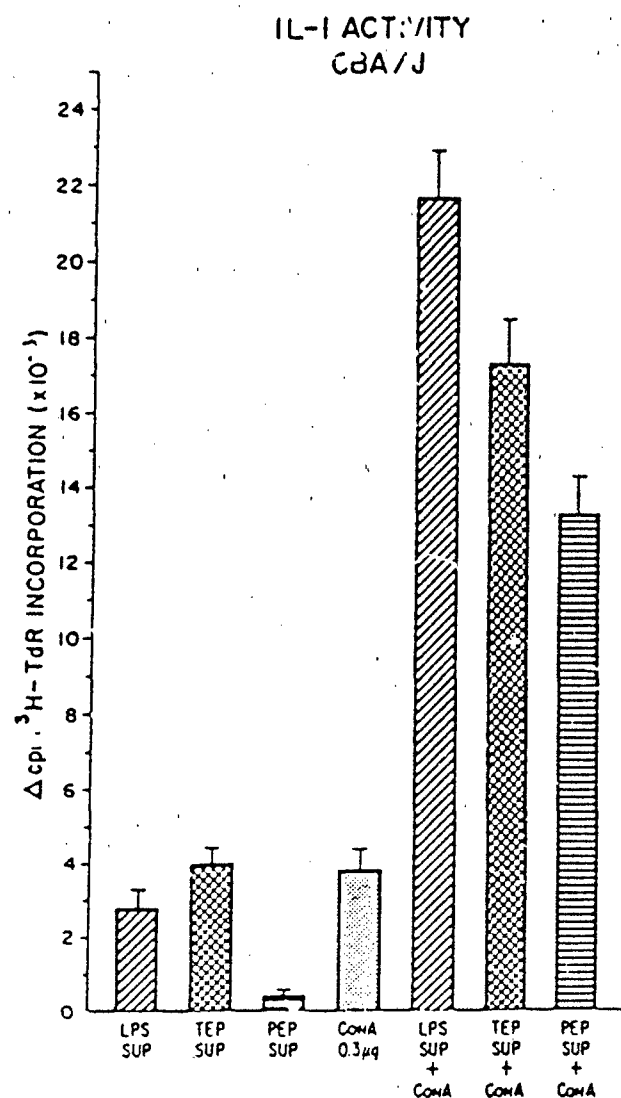


Figure 1. CBA/J peritoneal macrophages plated in the presence of 100 μg each of LPS, TEP or PEP for 24 hrs. The supernatants from each macrophage culture were added to C3H/HeJ thymocyte cultures in the presence or absence of Concanavalin A.

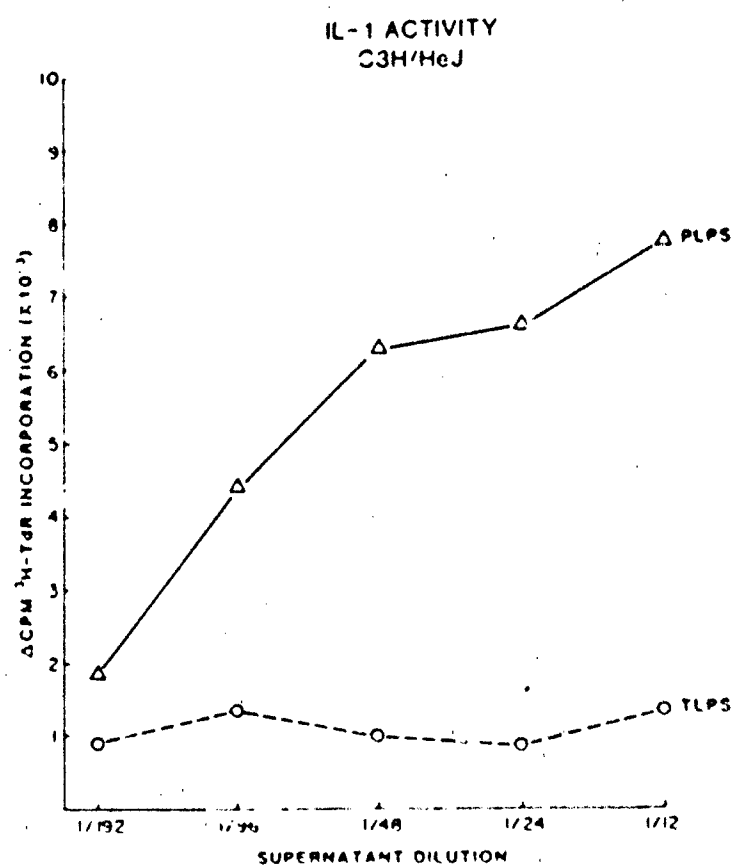


Figure 2. C3H/HeJ peritoneal macrophages cultured in the presence of 100  $\mu\text{g}$  of LPS for 24 hrs. The supernatants from these cultures were then added to C3H/HeJ thymocytes with Con A at a concentration of Con A which was insufficient to activate thymocytes alone.

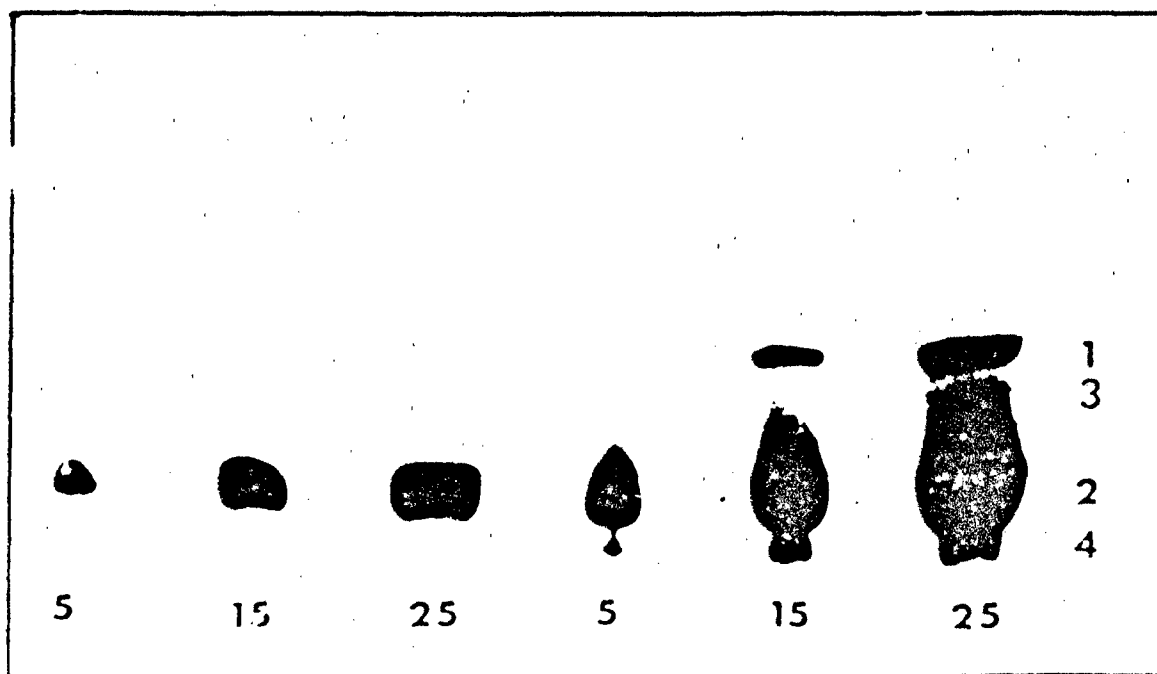


Figure 3. SDS-PAGE slab gel of pertussis LPS. The left three lanes are FW-LPS. The right three lanes are PCE-LPS at the concentrations shown in micrograms.

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